

Pax3 and Regulation of the Melanocyte-specific Tyrosinase-related Protein-1 Promoter*

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Previous work has established that the melanocyte-specific tyrosinase-related protein-1 (TRP-1) promoter is regulated positively by the microphthalmia-associated transcription factor Mitf, acting through the conserved M box and negatively by the T-box factor Tbx2, which can bind two “melanocyte-specific elements” termed the MSEu and MSEi. Both the MSEu and MSEi, which share a 6-base pair GTGTGA consensus, are also recognized by a previously unidentified melanocyte-specific factor, MSF. Here we show using a combination of DNA binding assays, proteolytic clipping, and anti-Pax3 antibodies that MSF is indistinguishable from Pax3, a paired homeodomain transcription factor implicated genetically in melanocyte development and the regulation of the *Mitf* promoter. Consistent with Pax3 being able to bind the TRP-1 promoter, Pax3 is expressed in melanocytes and melanomas, and TRP-1 promoter activity is up-regulated by Pax3. The results identify a novel role for Pax3 in the expression of TRP-1, and the potential role of Pax3 in the melanocyte lineage is discussed.

The development of the melanocyte lineage presents a fascinating opportunity to analyze the complex interplay between signal transduction pathways and transcription factors, which underlies development. Because melanocytes are not essential for viability and variations in pigmentation are obvious (1), over 70 independent genetic loci have been implicated in the development or function of these melanin-producing cells. Of the 20 or so that have been cloned to date, some, such as the genes encoding tyrosinase or tyrosinase-related protein-1 (TRP-1),¹ have a clearly defined function in the genesis of pigment. On the other hand, genes such as the endothelin B (2–4) and c-Kit receptors (5), and the microphthalmia (6–8), Sox10 (9, 10), and Pax3 (11) transcription factors have been implicated in the developmental pathway leading to the genesis of the mature pigment-producing melanocyte from a non-pigmented melanoblast precursor cell originating in the trunk neural crest.

Particularly interesting are mutations affecting the Pax3-paired homeodomain transcription factor, exemplified by the splotch allele (11), which expresses a truncated Pax3 protein. Although splotch homozygotes die *in utero*, heterozygous

splotch mice exhibit pigmentation defects resulting from the loss of a proportion of the melanoblasts migrating away from the neural crest. The loss of melanocytes in splotch mice may be explained by the fact that Pax3 has recently been shown to activate expression from the promoter for the gene encoding the microphthalmia-associated basic helix-loop-helix-leucine zipper transcription factor (Mitf) (12); mice devoid of functional Mitf lack all pigment cells, and a decrease in Mitf levels resulting from monoallelic loss of *Pax3* would account for the pigmentation defect exhibited by splotch mice. The ability of Pax3 to regulate expression of Mitf is paralleled by the role of Pax3 in skeletal muscle formation where it is required for expression of the basic helix-loop-helix transcription factors MyoD, Myf-5, and myogenin (13, 14), which play essential roles in myogenesis.

The role of Pax3 in regulating *mitf* expression, and consequently melanocyte development, appears to be relatively well defined, however, it is not known whether Pax3 might also play a role in differentiation of melanocytes as characterized by the expression of the genes involved in the manufacture of the pigment melanin, a process specific to this cell type.

We have previously characterized the cis-acting requirements for expression of the human tyrosinase and mouse TRP-1 promoters (15–17). Both promoters are dependent on the activity of Mitf, which acts through an initiator E box in the tyrosinase promoter (15) as well as via the highly conserved M box element (17, 18) present in the promoters for the tyrosinase, *TRP-1* and *TRP-2* genes. *TRP-1* expression is also regulated by two additional elements with the sequence GTGTGA termed the MSEu and MSEi, which appear to act as strong negative regulatory sequences (16, 19). Both the MSEu and MSEi are recognized by an unidentified factor termed MSF. However, point mutational analysis revealed that binding by MSF did not correlate to repression of the TRP-1 promoter, but rather may be involved in positive regulation of *TRP-1* expression (16). Instead, repression appears to correspond to binding by Tbx2 (19), a member of the T-box transcription factor family (20, 21) expressed in melanoblasts and melanocytes. If Tbx2 acts as the repressor of *TRP-1*, the question remained as to the nature of MSF.

Here we demonstrate, using a combination of proteolytic clipping and DNA binding assays that MSF is in fact Pax3. Moreover we demonstrate that Pax3 can activate TRP-1 expression in transfection assays and that Pax3 is expressed in melanocytes and melanomas. Thus Pax3, which plays an essential role early in melanocyte development, also regulates a marker of melanocyte differentiation, TRP-1.

MATERIALS AND METHODS

Cell Lines and Transfection Assays—The mouse melanoma cell line, B16, was grown in RPMI 1640 with 10% fetal calf serum. Transfections were performed using Fugene reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions. Cells were plated at 1×10^4 /24 wells/plate 24 h before transfection. A total of 600 ng of DNA was mixed with 1 μ l of Fugene in 60 μ l of serum-free medium, left for 15 min

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¹ The abbreviations used are: TRP-1, tyrosinase-related protein-1; ITT, *in vitro* transcribed/translated; MSF, melanocyte-specific factor.

at room temperature, and then added to the cells. 48 h post-transfection, cells were washed 2 times with cold phosphate-buffered saline and harvested using 100 μ l of lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol). Luciferase assays were performed using the Promega luciferase assay system with 20 μ l of cell extract according to the manufacturer's instructions. Luciferase activity was detected using a microplate luminometer apparatus (MicroLumat Plus, EG&G Berthold). All transfections were repeated using different amounts of DNA, and pCH110 containing the SV40 promoter driving expression of a LacZ reporter was used as an internal control for transfection efficiency (1 μ g/transfection).

Construction of Reporter Plasmids Used—The parental plasmid used for all luciferase assays was the pGL3-Basic vector (Promega). The TRP-1 promoter (−336/+114) and its mutated form LS-MSEu, described previously (16), were subcloned as *Xba*I/*Hind*III fragments into the pGL3 vector (*Nhe*I/*Hind*III). The MSEi.M3 mutant was isolated in three steps by polymerase chain reaction-based mutagenesis and was cloned as an *Xba*I/*Hind*III fragment in the pGL3 vector. Details of the precise cloning strategy used are available on request.

DNA Binding and Proteolytic Clipping Assays—The band shift assays were performed in a final volume of 20 μ l containing HEPES (pH 7.9), 10% glycerol, and 112 mM KCl. Nuclear extracts were prepared as described previously (16). *In vitro* transcribed/translated (ITT) protein was made according to the manufacturer's instructions (Promega TNT T7 Quick Coupled transcription). Nuclear extracts or ITT Pax3 were preincubated at 0 °C with 1 μ g of poly(dIdC-dIdC) for 10 min before the addition of 10, 50, or 250 ng of cold competitor DNA. After a further incubation period of 10 min, approximately 0.5 ng of oligonucleotide probe, labeled at each end by filling in 5' overhangs with Klenow polymerase and the appropriate [α - 32 P]dNTP, was added to the reaction for a further 20 min before loading onto an 8% polyacrylamide gel (44:1 acrylamide/bisacrylamide ratio) and electrophoresis at 200 V for 1.5 h.

The sequences of double-stranded oligonucleotides used as probes are as follows: MSEi, 5'-ctagaGAATTCACCTGGTGTGAGAAGGGATTAGTt-3'; MSEu, 5'-ctagaAAAGCTAACAGAAAATACAAGTGTGACATt-3'; Pax3, 5'-ctagaCACCGCACGATTAGCATCGTCACGCTTCAG-3'. Competitor sequences are described in the figures.

Proteolytic clipping (22, 23) was achieved by adding 10, 100, or 1,000 ng of trypsin or chymotrypsin, or V8 protease to the standard band shift reaction after 10 min of incubation with the probe and were loaded to the gel after a further 10 min of incubation at room temperature.

Anti-Pax3 Antibody—The specific anti-Pax3 antibody used in this study has been described previously (24) and was a kind gift from Dr. Martine Roussel (St. Jude Children's Research Hospital, Memphis, TN).

RESULTS

DNA Binding Specificity of MSF—In addition to the M box, the TRP-1 promoter is regulated by the MSEu and MSEi elements, which share a GTGTGA motif (16). This sequence is recognized both by the T-box factor Tbx2 (19) and by a factor found in all melanocyte and melanoma cell lines tested termed MSF (16). It was essential to establish the identity of MSF if the regulation of TRP-1 was to be understood. As a first step, we examined the precise requirements for sequence recognition by MSF by using a series of oligonucleotides (Fig. 1A) bearing specific substitutions in the MSEu and MSEi elements. These oligonucleotides were used as competitors in DNA binding band shift assays using either an MSEu or MSEi probe. Using an MSEu probe, and B16 melanoma cell nuclear extract, a specific complex corresponding to MSF was observed as described previously (Fig. 1B). MSF binding was efficiently competed by the MSEu and also by the MSEi. A point mutation, pm1, affecting the first base of the GTGTGA motif severely reduced binding by MSF. Binding was essentially abolished by mutations at positions 3 and 4 of the MSEu (pm3 and pm4, respectively), and severely reduced (at least 25-fold) using pm2, pm5, and pm6, in which bases 2, 5, and 6 of the MSEu are mutated. Thus, mutation of any of the bases within the MSEu severely reduces binding by MSF.

We next examined more precisely the requirements for binding the MSEu by using competitors in which specific residues were substituted by methylated bases or inosine (Fig. 1C). In the MSEu.CI competitor, each T residue is substituted with a C

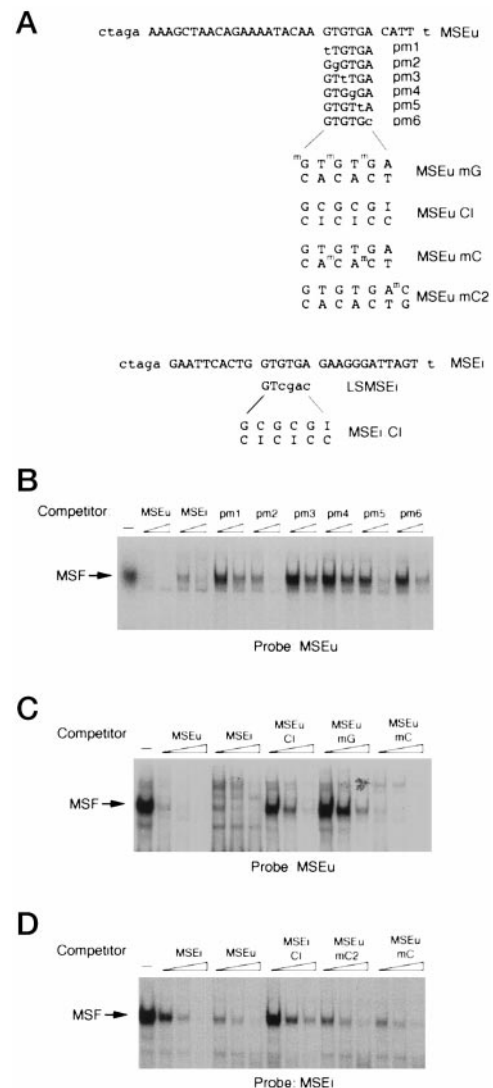


FIG. 1. MSF DNA binding specificity. A, oligonucleotides used as probes and competitors. All oligonucleotides used contain additional bases at each end indicated in lowercase letters to facilitate cloning. The MSEu and MSEi GTGTGA motifs are overlined. The derivatives used in the competition assays are identical except for the indicated residues shown in lowercase letters. mG indicates a methylated G residue, and mC a methylated C residue. I indicates inosine and lowercase within these elements indicates base substitutions. B–D, band shift assays using the indicated probes and competitors at either 50 and 250 ng (B), or 10, 50, and 250 ng (C and D).

residue, whereas the inosine substitutes for A. The result is a mutant MSEu in which specific changes have been introduced into the major groove, although leaving the minor groove unchanged. Given the severity of the changes to the major groove, we might have expected the MSEu.CI site not to bind MSF. However, MSF retained the ability to bind the MSEu.CI oligonucleotide but around 5-fold less efficiently than the wild type MSEu. In contrast binding to an MSEu in which each G residue was methylated (MSEu.mG) reduced MSF binding by more than 25-fold, indicating that the presence of methyl groups in the major groove of the top strand severely affected binding by MSF. Surprisingly, on the other hand, methylation of two C residues on the bottom strand (MSEu.mC2), failed to affect binding by MSF. Taken together these data provide an indication that MSF binds asymmetrically in the major groove with the presence of methyl groups on the top strand preventing DNA binding, whereas methyl groups on the bottom strand have no effect.

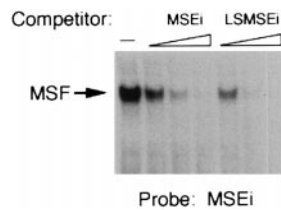


FIG. 2. **MSF binds the LSMSEi mutant.** Band shift assays using MSEi probe and the indicated competitors at 10, 50, and 250 ng. The sequence of the LSMSEi mutation is shown in Fig. 1A.

Using the MSEi as a probe (Fig. 1D), we were also able to show that a similar substitution of T with C, and A with inosine within the MSEi (MSEi.CI), had only a minor effect on binding by MSF. As with the MSEu probe, binding by MSF to the MSEi was also efficiently competed by an oligonucleotide where two C residues on the bottom strand were methylated (MSEu.mC) and where a 3'-flanking C residue was methylated (MSEu.mC2). The results obtained for binding to the MSEi probe are therefore entirely consistent with those obtained using the MSEu probe.

MSF Binding to the MSEi Requires an Additional 3' Element—The data obtained for the MSEu suggested that MSF bound asymmetrically within the major groove and that each base within the MSEu was essential for MSF binding. We have previously described a mutation of the TRP-1 promoter in which 4 bases within the MSEi are altered (16). This mutation, termed LSMSEi, results in up to an 80-fold increase in TRP-1 promoter activity in either melanoma or melanocyte cell lines (16, 19). Consistent with Tbx2 acting as a repressor of TRP-1 expression, Tbx2 is unable to bind the LSMSEi mutant (19). In contrast, binding by MSF is relatively efficient, being only around 5-fold reduced compared with a wild type MSEi (Fig. 2). The result was surprising, because although each base of the MSEu was important for MSF binding, mutation of 4 bases within the MSEi failed to affect binding by MSF more than 5-fold. One possible explanation was that binding to the MSEi required sequences outwith the core GTGTGA motif. In an attempt to identify any such auxiliary binding site, we introduced additional mutations into the core MSEi GTGTGA motif as well as the flanking sequences. The mutants used are shown in Fig. 3A, and the results of the DNA binding assays obtained using these mutant forms of the MSEi as competitors is shown in Fig. 3B. As shown above, binding of MSF to the MSEi is competed by the LSMSEi mutant around 3–5-fold less efficiently than the wild type MSEi. Introduction of mutations into sequences 5' to the GTGTGA motif (mutants M1 and M2) failed to affect binding by MSF. In contrast, mutation of an AT-rich sequence 3' to the MSEi in mutant M3 resulted in greatly reduced MSF binding by around 25-fold, indicating that this region may represent the anticipated auxiliary MSF recognition element. Mutation of the first 2 bases of the MSEi in mutant M4 reduced binding by around 3-fold, whereas a mutation affecting the same bases together with the 3 bases immediately 3' to the GTGTGA motif again inhibited binding by MSF by around 25-fold. However, the M6 mutant, which affects the 3'-flanking sequence alone, binds MSF with only around a 2-fold reduction in efficiency.

In summary, the entire series of DNA binding assays would indicate that at the MSEu each base is important for binding with asymmetric recognition of the major groove, whereas at the MSEi, although bases within the GTGTGA motif are important, a significant contribution to binding is made at the 3'-flanking sequences, most notably by the AT-rich motif affected by the M3 mutation. This pattern of DNA recognition is extremely reminiscent of DNA binding by members of the

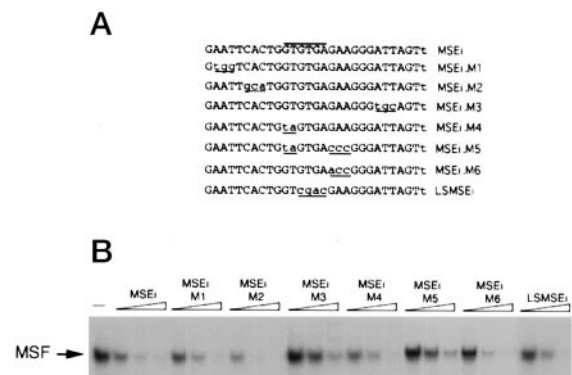


FIG. 3. **MSF binding to the MSEi.** A, the sequences of the probes and competitors used with the MSEi overlined and mutations indicated as underlined lowercase letters. B, band shift assay using indicated probes and competitors at 10, 50, and 250 ng.

paired homeodomain family, which play key regulatory roles during development (for review, see Ref. 25). DNA recognition by the paired domain is complex, with different paired domains able to recognize different though related sequences. From the crystal structure of the *Drosophila* protein Prd (26), it is nevertheless evident that the effects of mutations introduced into the MSEu would be consistent with recognition of this motif by a paired domain, whereas the homeodomain (27), which can cooperate in DNA binding with the paired domain (28), would be able to target the AT-rich motif 3' to the MSEi GTGTGA element.

Pax3 Is Expressed in Melanocytes and Melanomas—If MSF were indeed a member of the paired homeodomain family of transcription factors, the most likely candidate would be Pax3, which has been implicated genetically in the regulation of melanocyte development, both in Splotch mice (11) and in human Waardenburg syndrome type 1 (29, 30). However, the genetic defect associated with loss of Pax3 might reflect loss of melanoblast precursor cells, rather than a specific failure of Pax3 to regulate gene expression after commitment to the melanocyte lineage. Moreover, although ectopic expression of Pax3 can regulate the Mitf promoter and bind the promoter *in vitro*, surprisingly, it had not previously been determined whether Pax3 is in fact expressed in cells of the melanocyte lineage. Thus, before attempting to determine whether MSF was related to Pax3, it was essential to establish that Pax3 was indeed expressed in melanocytes. We therefore performed a Western blot using the mouse melanocyte cell line melan-a, as well as the mouse B16 and human 501 melanoma cell lines and probed with a specific anti-Pax3 antibody. ITT Pax3 was used as a control. The results (Fig. 4) indicate that Pax3 is expressed in both the melanocyte and melanoma cell lines, but not in the unrelated 3T3 cell line, a result confirmed both by reverse transcription-polymerase chain reaction and Northern blotting (data not shown).² The absence of Pax3 in 3T3 cells is in agreement with our previous work where MSF DNA binding activity was not detected in 3T3 cells (16). The additional faster migrating band observed using the B16 melanoma cell line may represent a degradation product of Pax3.

MSF and Pax3—The fact that Pax3 is expressed in melanocytes and melanoma cells added weight to the argument that MSF and Pax3 were related. Significantly, DNA binding site selection for high affinity Pax3 recognition sequences (31) identified a number of sequences with very strong homology to the MSEu or MSEi including for example AAGTGTGAC, identical to the MSEu over 9 base pairs, and an 8-base pair sequence

² Dot Bennett, St. George's Hospital Medical School, London, personal communication.

identical to the MSEi, TGGTGTGA, which also was located a short distance upstream from an AT-rich element. Taken together with the fact that Pax3 is expressed in cells of the melanocyte lineage, the DNA binding data were consistent with MSF being Pax3. In addition, by using *in vitro* tran-

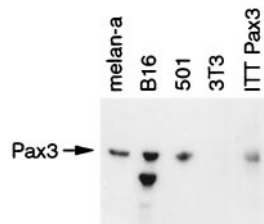


FIG. 4. Pax3 is expressed in melanocytes and melanoma cell lines. Western blot using anti-Pax3 antibody and either the melanocyte cell line, melan-a, or the mouse B16 and human 501 melanoma cell lines. Also shown are 3T3 cells, used as a negative control, and ITT Pax3 as a positive control. An equivalent amount of total protein was loaded for all cell lines.

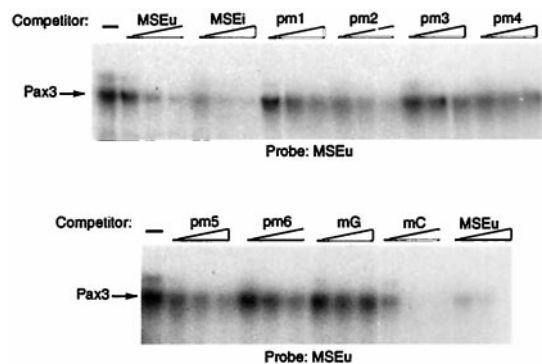


FIG. 5. Pax3 DNA binding specificity. Band shift assay using ITT Pax3 and the indicated probes and competitors corresponding to those shown in Fig. 1A. Competitors were used at 10, 50, and 250 ng.

scribed/translated Pax3 in a band shift assay (Fig. 5) together with the MSEu probe and competing with a selection of the oligonucleotides used to determine the DNA binding specificity of MSF shown in Fig. 1, it was evident that Pax3 and MSF recognized DNA in a very similar fashion. Thus for example, Pax3 could recognize both the MSEu and MSEi elements, was less affected by the pm2 mutation than the other point mutations in the MSEu, and bound the mC oligonucleotide but not the mG competitor, indicating that like MSF, DNA binding by Pax3 was differentially affected by methylation of the top or bottom strands of the MSEu binding site.

We also used probes corresponding to either a consensus Pax3 binding site or the MSEu or MSEi elements to show that Pax3 could recognize the TRP-1 promoter sequences (Fig. 6A). No binding was observed using unprogrammed ITT reaction (not shown).

We next chose to use an alternative approach to investigate more closely the identity of MSF. To this end, we made use of a proteolytic clipping assay that is used to identify highly related DNA-binding proteins (22) and has been used by us previously to identify the Brn-2 transcription factor in melanoma cells (23). In this assay, nuclear extract or *in vitro* transcribed/translated protein is subjected to increasing concentrations of a proteolytic enzyme and specific cleavage products, which retain the ability to bind DNA are detected using a band shift assay and an appropriate radiolabeled probe. The pattern of DNA bound cleavage products obtained is highly specific for a given protein, being dependent not only on the precise position of specific protease cleavage sites in the primary amino acid sequence but also on their relative accessibility within the protein, which is dictated by the protein conformation. A specific pattern of DNA bound products is therefore diagnostic of a particular protein.

To investigate the possibility that MSF and Pax3 were identical, we initially performed band shift assays using a consensus Pax3 binding site as probe and either ITT Pax3 or B16 cell nuclear extract to assess whether Pax3 DNA binding activity

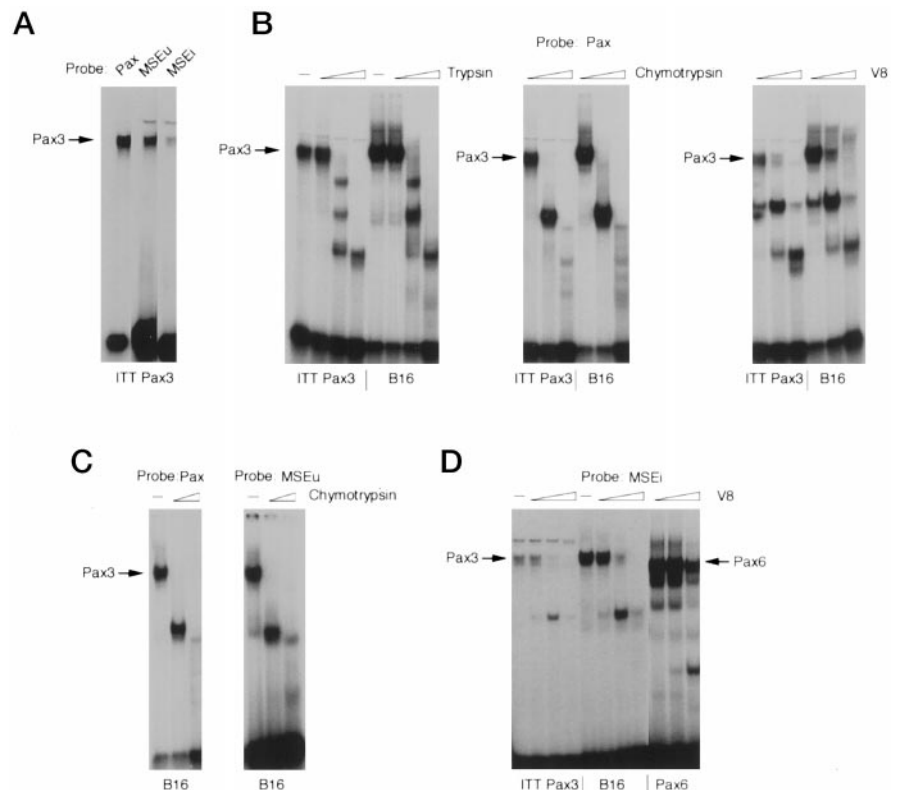


FIG. 6. MSF and Pax3. Proteolytic clipping band shift assay using ITT Pax3, baculovirus-expressed Pax6, or B16 melanoma nuclear extract, and the indicated probes and proteases. The concentration of the proteases used was determined empirically to yield partial proteolysis at increasing concentrations. The full sequences of the Pax3, MSEi, and MSEu probes are shown under "Materials and Methods."

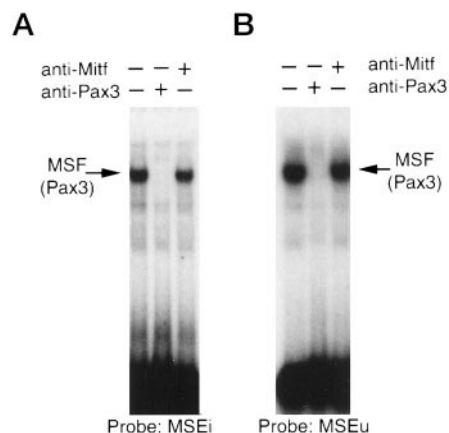


FIG. 7. MSF is recognized by anti-Pax3 antibody. Band shift assays using the indicated MSEi (A) or MSEu (B) probes and B16 melanoma cell nuclear extract. Extract was incubated with either the anti-Pax3 antiserum or a control anti-Mitf anti-serum for 30 min before the addition of the probe.

was present in B16 nuclear extract. After allowing the protein to bind the probe, the DNA binding reactions were treated with limited amounts of either trypsin, chymotrypsin, or V8 protease. The results obtained are presented in Fig. 6B and demonstrate clearly that B16 nuclear extracts contain Pax3: first, the relative migration of the intact complex obtained using ITT Pax3 and B16 extract is identical; and second, the pattern of DNA binding complexes obtained following proteolytic treatment using any of the three proteases is identical when comparing ITT Pax3 to B16 extract. Because the results from the proteolytic DNA binding assays indicate that Pax3 is present in the B16 melanoma cell nuclear extracts, we next compared the pattern of bands obtained using a consensus Pax probe to those obtained using an MSEu probe together with B16 cell nuclear extract and chymotrypsin cleavage (Fig. 6C). Again, the relative migration and pattern of both the intact and proteolytically cleaved bands obtained with the Pax and MSEu probes is identical, and the same as that obtained using ITT Pax3 (compare with Fig. 6B), strongly suggesting that the MSEu is recognized by Pax3.

The specificity of this assay is highlighted by the fact that the highly related paired homeodomain factor Pax6 can bind the MSEi probe, but the Pax6 MSEi complex migrates differently from those containing MSF or Pax3, and moreover the V8 cleavage pattern is different for Pax6 (Fig. 6D) but identical when using Pax3 or MSF.

Taken together, the results obtained from the DNA binding and proteolytic clipping assays are consistent with MSF and Pax3 being identical.

To confirm that MSF and Pax3 were indeed the same, we made use of the specific anti-Pax3 antibody used for the Western blot shown in Fig. 4, in a bandshift assay using either an MSEi or MSEu probe and B16 cell nuclear extract. The results shown in Fig. 7 demonstrate that DNA binding by MSF to either probe was strongly inhibited by the anti-Pax3 antibody, but was unaffected using an anti-Mitf antibody that we have used in similar assays to inhibit binding by Mitf to the M box (not shown). Thus, both the proteolytic clipping assays as well as the antibody supershifts are consistent with MSF and Pax3 being identical.

Pax3 Regulates the TRP-1 Promoter—If MSF and Pax3 are the same, then we might expect Pax3 to regulate transcription from the TRP-1 promoter. To address this question, we transfected B16 melanoma cells with a TRP-1 luciferase reporter extending between -336 and $+114$ (Fig. 8A) either alone or together with a vector expressing Pax3. The results obtained

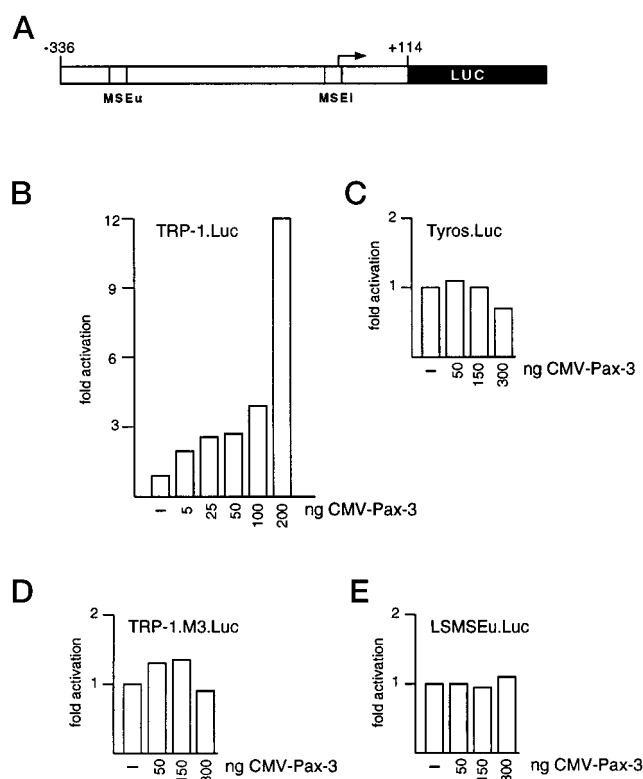


FIG. 8. Pax3 can activate the TRP-1 promoter. A, schematic diagram showing the TRP-1-luciferase reporter used. B, the wild type TRP-1 luciferase reporter (300 ng) was transfected into B16 melanoma cells either alone or together with the indicated amounts of a cytomegalovirus-Pax3 expression vector and assayed for luciferase activity 48 h post-transfection. C-E, the same as for panel B, but using either a tyrosinase-luciferase reporter (C) or the full-length TRP-1 promoter containing either the MSEi.M3 mutation (D) (see Fig. 2A) or the LSMSEi mutation (E).

demonstrated that increasing the amount of Pax3 expression plasmid used in the transfection resulted in increasing TRP-1 promoter activity (Fig. 8B) with up to 12-fold activation being achieved at the highest amount of Pax3 expression vector used. Activation of TRP-1 was specific because no activation of a tyrosinase-luciferase reporter was observed (Fig. 8C), consistent with the fact that the tyrosinase promoter lacks binding sites for Pax3(MSF). To ask whether the MSEu or MSEi were required for activation by Pax3, we also used reporters in which the MSEu or MSEi had been mutated. Specifically, the MSEi mutation used was that affecting the auxiliary Pax3 recognition site, MSEi.m3, because this mutation does not affect binding by Tbx2; the MSEu mutant, LSMSEu, fails to bind either Pax3 or Tbx2 and was used, because we have yet to identify point mutations that distinguish between binding by these two proteins at the MSEu. In contrast to the wild type TRP-1 promoter, which was activated by Pax3, neither the MSEi.M3 nor the LSMSEu mutant was affected even at the highest doses of Pax3 expression vector (Fig. 8, D and E). We conclude that Pax3 can activate the TRP-1 promoter but that efficient activation appears to require both the MSEu and MSEi.

Because Pax3 and Sox10, an HMG box protein, have been reported to activate transcription synergistically in glial cells (32) and because Sox10, as well as Pax3, is implicated in melanocyte development (9, 10), we also asked whether Sox10 expression could affect the activation of TRP-1 by Pax3. The TRP-1 luciferase reporter was transfected into B16 melanoma cells together with different ratios of vectors expressing Pax3 and Sox10. In no experiment were we able to observe any

cooperativity between Pax3 and Sox10 on the TRP-1 promoter (data not shown).

DISCUSSION

We have previously established that the TRP-1 promoter is regulated by a combination of positive and negative elements (16, 17). One positive element, the M box, is targeted by *Mitf* (33), whereas two additional elements, termed the MSEu and MSEi, are recognized by the T-box factor Tbx2 and a previously unidentified DNA-binding protein known as MSF (16, 34). If the regulation of the TRP-1 promoter was to be fully understood, it was important to establish the identity of MSF. Here we show, using a combination of proteolytic clipping and DNA binding assays as well as by using a specific anti-Pax3 antibody, that MSF and Pax3 appear to be identical, and Pax3 can up-regulate TRP-1 promoter activity in co-transfection assays. We also demonstrate for the first time that Pax3 is expressed in melanocytes and melanoma cells.

Pax3 has already been identified genetically as playing an essential role in melanocyte development; mutations in Pax3 can give rise to the Splotch phenotype in mice (11) or Waardenburg's syndrome type-1 in humans (29, 35, 36). Both Splotch and Waardenburg's syndrome type-1 are characterized by a partial loss of neural crest-derived melanocytes that may be accounted for, at least in part, by a requirement for Pax3 for the expression of the gene encoding *Mitf* (12). However, the ability of Pax3 to bind and activate the TRP-1 promoter suggests an additional role for Pax3 in the regulation of melanocyte differentiation. Although the MSEu and MSEi can act as negative regulatory elements, the experiments presented here suggest that Pax3 may function as a positive regulator of TRP-1 expression. For example, the LSMSEi mutation can result in up to an 80-fold increase in TRP-1 promoter activity (16, 34), but this mutation failed to affect binding by Pax3 more than around 5-fold, whereas transfection of a Pax3 expression vector resulted in increased expression from a reporter gene driven by the TRP-1 promoter. Consistent with Pax3 not being responsible for repression of the TRP-1 promoter in melanocyte or melanoma cell lines, previous point mutational analysis of the MSEu and MSEi demonstrated that recognition of the MSEu and MSEi by Tbx2 correlated with transcriptional repression (34). Taken together, these data suggest that at the MSEu and MSEi, Tbx2 may repress and Pax3 activate TRP-1 expression. In addition, although Tbx2 and Pax3 DNA binding specificity are distinct, for example Pax3 but not Tbx2 can bind the LSMSEi mutant, they clearly require overlapping sequences. As such it seems likely that binding by Pax3 and Tbx2 is mutually exclusive. What determines whether any given binding site is recognized by Pax3 or Tbx2 at any particular time will be determined by several factors including, the relative concentrations of each factor within the cell, and the nature of any regulation dictated by the activity of specific signal transduction pathways. At the moment, virtually nothing is known of the factors governing the activity or expression of either Tbx2 or Pax3.

We have shown here that Pax3 is expressed in melanocytes as well as melanoma cell lines. Northern blot analysis² has also established that Pax3 is expressed both in melanoblasts and in cells that have the characteristics of melanoblast precursors. *TRP-1*, and *Mitf*, on the other hand are expressed in both melanoblasts and melanocytes, but are not expressed before commitment to the melanocyte lineage. Thus during development, the expression of Pax3 alone is clearly insufficient to allow *TRP-1* or *Mitf* to be expressed. Because Pax3 has also been demonstrated to up-regulate the *Mitf* promoter, some mechanism must operate to prevent Pax3 from inappropriately activating the *Mitf* and *TRP-1* promoters in melanoblast pre-

cursor cells. One possibility is that in melanoblast precursor cells, Pax3 lacks an essential cofactor to enable it to activate transcription. Alternatively, because Pax3 has been shown to possess domains that mediate either transcription activation or transcription repression (37), it is also possible that Pax3 acts to repress transcription in pre-melanoblasts, but activates transcription after the transition to a melanoblast. Such a switch would require either that Pax3 is regulated by specific signal transduction pathways and/or that there is selective recruitment of co-factors to Pax3 to mediate its transcription activation/repression functions. Although it is not known how Pax3 is regulated, it has recently been shown that Pax3 can interact with HIRA, a factor implicated in chromatin modulation and a homologue of *Saccharomyces cerevisiae* transcriptional co-repressors (38). This observation would suggest that one role of Pax3 is to organize the chromatin structure across Pax3 target promoters, though whether the interaction between Pax3 and HIRA results in a positive or negative regulation of transcription is unclear.

It is also possible that it is the level of Pax3 expression *per se* that is the critical factor with the amount of Pax3 protein present in a cell needing to exceed a threshold before activation of the *Mitf* promoter can occur. This may be particularly relevant because melanoblasts express higher levels of Pax3 than melanoblast precursors.² This situation appears to occur during muscle development where Pax3 is required for the expression of MyoD (13, 14). Particularly interesting is the observation that whereas cells derived from dissociated neural tube normally express Pax3, they are induced to undergo myogenesis by infection with a retrovirus expressing Pax3 (14). This result suggests that the ability of Pax3 to induce myogenesis is normally suppressed by an inhibitor and that elevating Pax3 levels overcomes repression and leads to myogenesis. The nature of the repressor is unknown, but it may be that a similar mechanism operates to prevent Pax3 from inducing TRP-1 or *Mitf* expression in melanoblast precursor cells. Our future work will attempt to address this issue.

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Pax3 and Regulation of the Melanocyte-specific Tyrosinase-related Protein-1 Promoter

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